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(54) **MODIFIED POLYMERASES FOR
REPLICATION OF THREOSE NUCLEIC
ACIDS**

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(57) **ABSTRACT**

Methods and compositions for replication of threose nucleic
acids (TNAs) are described. The described methods include
a method for transcribing a DNA template into a TNA, and
a method for reverse transcribing a threose nucleic acid into
a cDNA.

6 Claims, 6 Drawing Sheets

FIG. 1

20 40 60
9N-RI atgattctggataccgattacattaccgaaaacggcaaacgggtgattcgtgtgttcaaa
M I L D T D Y I T E N G K P V I R V F K
80 100 120
9N-RI aaagaaaacggcgaattcaaaatcgaatacgcgtacctttgaaccgtatttttacgcg
K E N G E F K I E Y D R T F E P Y F Y A
140 160 180
9N-RI ctgctgaaagatgatagcgcgatcgaagatgtgaaaaaagtgaccgcgaaacgtcatggc
L L K D D S A I E D V K K V T A K R H G
200 220 240
9N-RI accgtggtgaaagtgaacggtgcggaaaaaagtgcagaaaaaatttctgggcggtccgatt
T V V K V K R A E K V Q K K F L G R P I
260 280 300
9N-RI gaagtgtggaacgtgtatttcaaccatccgcaggatgtgccggcgattcgtgatcgtatt
E V W K L Y F N H P Q D V P A I R D R I
320 340 360
9N-RI cgtgcgcacccggcggtggatatttatgaatatgatataccggttcgcgaaacgttat
R A H P A V V D I Y E Y D I P F A K R Y
380 400 420
9N-RI ctgattgataaaggccctgattccgatggaaggcgatgaagaactgaccatgctggccctt
L I D K G L I P M E G D E E L T M L A F
440 460 480
9N-RI gcgattgcgaccctgtatcacgaaggcgaagaatttggcaccggcccgattctgatgatt
A I A T L Y H E G E E F G T G P I L M I
500 520 540
9N-RI agctatgcggatggcagcgaagcgcgtgtgattacctggaaaaaatcgatctgccgtat
S Y A D G S E A R V I T W K K I D L P Y
560 580 600
9N-RI gtggatgtggtagcaccgaaaaaagaaatgatcaaacgctttctgcgtgtgggtgcgtgaa
V D V V S T E K E M I K R F L R V V R E
620 640 660
9N-RI aaagatccggatgtgctgattacctataacggcgataactttgatttcgcgtatcigaaa
K D P D V L I T Y N G D N F D F A Y L K
680 700 720
9N-RI aaacggttcgaagaactgggcacatcaaatctaccctgggcccgtgatggtagcgaaccgaaa
K R C E E L G I K F T L G R D G S E P K
740 760 780
9N-RI attcagcgtatgggcgatcgttttgcgggtggaagtgaaggccgtattcattttgatctg
I Q R M G D R F A V E V K G R I H F D L
800 820 840
9N-RI tatccgggtgattcgcggtaccattaacctgccgacctataccctggaagcgggtgatgaa
Y P V I R R T I N L P T Y T L E A V Y E

FIG. 1 (continued)

860 880 900
9N-RI gcgggtgtttggcaaacgaaagaaaaagtgtacgcggaagaaattgcgcagggcgiggaa
A V F G K P K E K V Y A E E I A Q A W E

920 940 960
9N-RI agcggcggaaggcctggaacgtgtggcgcggttatagcatgggaagatgcgaaagtgacctat
S G E G L E R V A R Y S M E D A K V T Y

980 1,000 1,020
9N-RI gaactggggccgtgaatttttcccgatggaagcgcagctgtctcgtctgattggccagagc
E L G R E F F P M E A Q L S R L I G Q S

1,040 1,060 1,080
9N-RI ctgtgggatgtgagccgttagcagcaccggcaacctgggtggaatgggtttctgtcgtgctaaa
L W D V S R S S T G N L V E W F L L R K

1,100 1,120 1,140
9N-RI gcgtataaacgtaacgaactggccccgaacaaaccggatgaacgtgaactggcccgctcgt
A Y K R N E L A P N K P D E R E L A R R

1,160 1,180 1,200
9N-RI cgtggcggttatgccccgggttatgtgaaagaaccggaaacgtggcctgtgggataacatt
R G G Y A G G Y V K E P E R G L W D N I

1,220 1,240 1,260
9N-RI gtgtatctggattttcgtagccctgtatccgagcattattatcacccataacgtgagcccg
V Y L D F R S L Y P S I I I T H N V S P

1,280 1,300 1,320
9N-RI galacctgaaccgtgaaggcctgcaagaaatgatgtggcgccgggaagtgggccataaa
D T L N R E G C K E Y D V A P E V G H K

1,340 1,360 1,380
9N-RI ttctgcaagattttccggggcctttattccgagccctgtctggcgcatctgtctggaagaacgc
F C K D F P G F I P S L L G D L L E E R

1,400 1,420 1,440
9N-RI cagaaaatcaaacgcaaaatgaaagcgaccgttgatccgctggaaaaaaaaactgctggat
Q K I K R K M K A T V D P L E K K L L D

1,460 1,480 1,500
9N-RI tatcgtcagcgccgtattaaaaattctggccaacagcttctatggctattatgggttatggg
Y R Q R R I K I L A N S F Y G Y Y G Y A

1,520 1,540 1,560
9N-RI aaagcgcggttggtattgcaagaaatgcgcggaaagcgtgaccgcgtggggccgtgaatat
K A R W Y C K E C A E S V T A W G R E Y

1,580 1,600 1,620
9N-RI atcgaaatgggtgatccgcgaactggaagaaaaattcggccttcaaagtgcgtgtatgcggat
I E M V I R E L E E K F G F K V L Y A D

1,640 1,660 1,680
9N-RI accgatggcctgcaigcgaccattccgggtgaggatgcggaaaccgtgaaaaaaaaagcg
T D G L H A T I P G A D A E T V K K K A

FIG. 1(continued)

1,700 1,720 1,740
9N-R1 aaagaattcctgaaatacaatcaatccgaaactgccgggacctgctggaactggaatatgaa
K E F L K Y I N P K L P G L L E L E Y E

1,760 1,780 1,800
9N-R1 ggcttttatgtgctggcttttttcgtgacccaaaaaaaaatacgcgggtgatcgatgaagaa
G F Y V R G F F V T K K K Y A V I D E E

1,820 1,840 1,860
9N-R1 ggcasaattaccaccctggcctggaaattgtgctgctgattggagcgaaattgcgaaa
G K I T T R G L E I V R R D W S E I A K

1,880 1,900 1,920
9N-R1 gaaacccaggcgctgtgctggaagcgattctgaaacatggcgatgtggaagaagcggtg
E T Q A R V L E A I L K H G D V E E A V

1,940 1,960 1,980
9N-R1 cgtattgtttaagaagtgaccgaaaaactgagcaaatatgaagtccgccggaaaaactg
R I V K E V T E K L S K Y E V P P E K L

2,000 2,020 2,040
9N-R1 gtgattcatattcaaataccctgatctgctgattataaagcgaccggtccgcattgtg
V I H I Q I T R D L R D Y K A T G P H V

2,060 2,080 2,100
9N-R1 gcgggtggcaaacgtctggcagcgctggcgtgaaaattcgtccgggcaccgtgattagc
A V A K R L A A R G V K I R P G T V I S

2,120 2,140 2,160
9N-R1 tatattgtgctgaaaggcagcgccgtattggcgatcgtgcgattccggcggaatgaattt
Y I V L K G S G R I G D R A I P A D E F

2,180 2,200 2,220
9N-R1 gatccgaccaaaccatcgttatgatgcggaatattatatcgaaaaccagggtgctgccggcg
D P T K H R Y D A E Y Y I E N Q V L P A

2,240 2,260 2,280
9N-R1 gtggaacgtattctgaaagcgittggctatcgtaagaagatctgcgctatcagaaaacc
V E R I L K A F G Y R K E D L R Y Q K T

2,300 2,320
9N-R1 aaacaggiggggcctggggcgctggctgaaagttaaaggcaaaaaa (SEQ ID NO:2)
K Q V G L G A W L K V K G K K (SEQ ID NO:1)

FIG. 2A

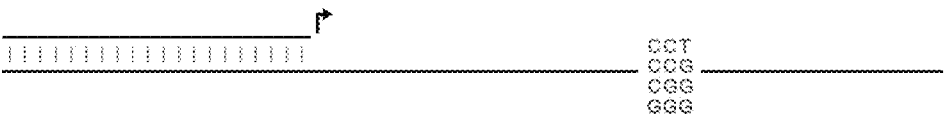


FIG. 2B

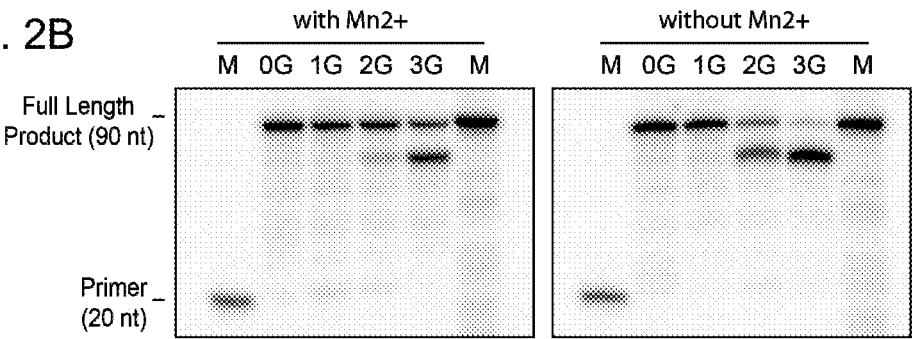


FIG. 3

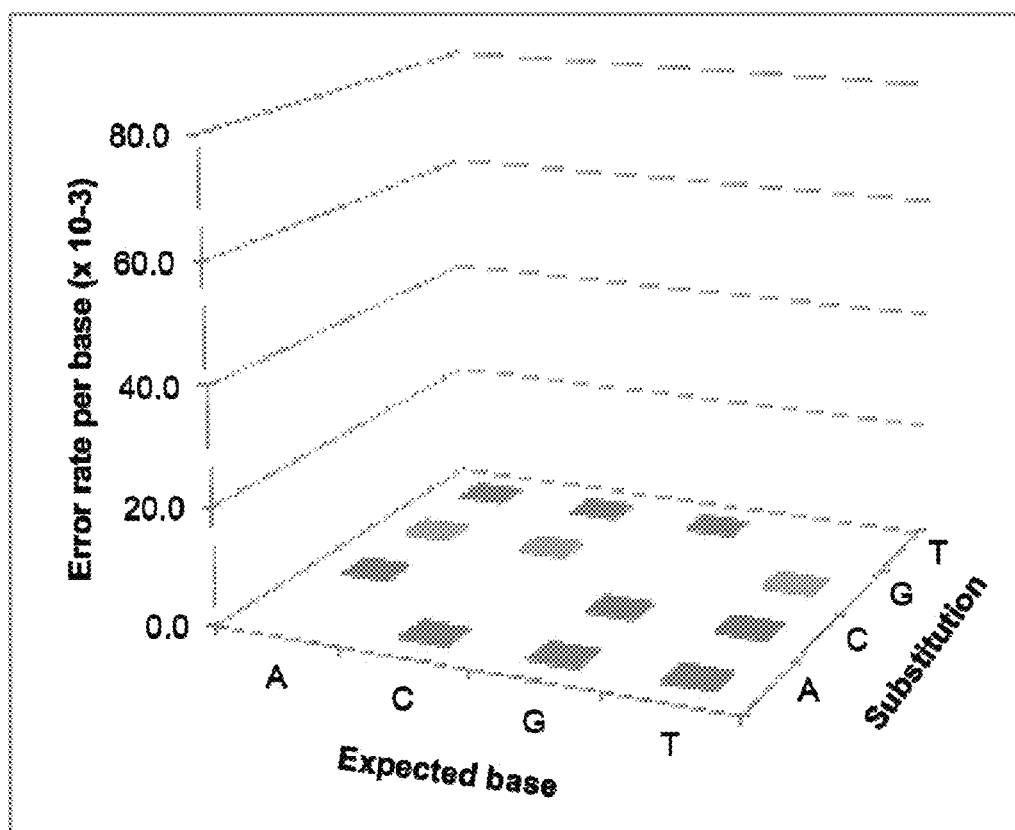
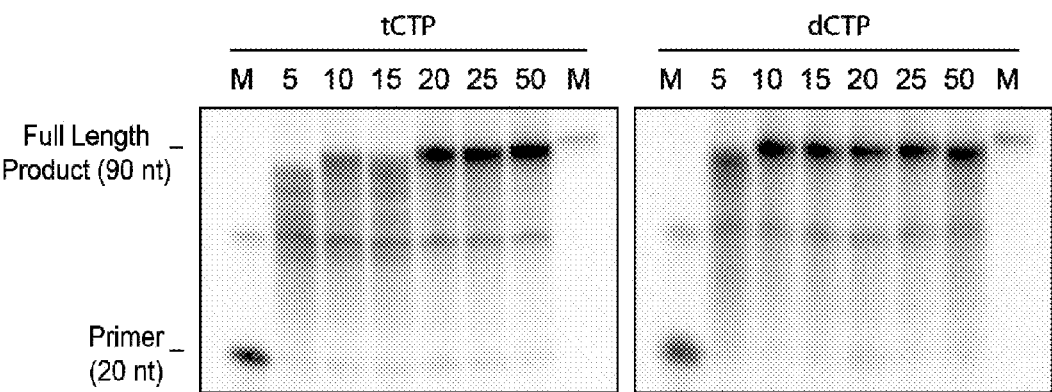


FIG. 4



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MODIFIED POLYMERASES FOR REPLICATION OF THREOSE NUCLEIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/038,975, filed Aug. 19, 2014, which is incorporated by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND

The emerging field of synthetic genetics provides an exciting opportunity to explore the structural and functional properties of synthetic genetic polymers by in vitro selection. However, achieving the goal of artificial genetics requires the ability to synthesize unnatural nucleic acid substrates ("XNA"s), such as threose-nucleic acids ("TNAs"), that are not otherwise available. Limiting this process, however, is the availability of enzymes and conditions that allow for the storage and propagation of genetic information present in unnatural nucleic acid polymers such as TNAs.

Threose nucleic acid (TNA) is an unnatural genetic polymer composed of repeating units of α -L-threofuranosyl nucleic acids with 2'3'-phosphodiester linkages. In 2001, TNA was shown to form stable antiparallel Watson-Crick duplexes with complementary strands of DNA and RNA, which provides a mechanism for exchanging genetic information with DNA and RNA. The structure of an all-TNA duplex, indicates that TNA is structurally similar to A-form RNA.

The discovery of TNA as an alternative genetic polymer with an RNA-like structure inspired other laboratories to begin developing the methodology needed to explore the functional properties of TNA by in vitro selection. Much of the early work in this area focused on identifying polymerases that could recognize TNA either in the template or as a nucleoside triphosphate. From these studies, we identified several DNA polymerases that could synthesize short sequences of DNA on a TNA template and other polymerases that could copy limited stretches of TNA on a DNA template. Herdewijn reported similar findings for the transcription of tTTP on a DNA template using thermophilic polymerases.

While these results show that TNA is not easily recognized by natural enzymes, subsequent screening did lead to the discovery of Terminator DNA polymerase, an engineered form of the Archeal family B replicative DNA polymerase isolated from the *Thermococcus* species 9° N. Relative to the natural enzyme, Terminator contains the mutations D141A and E143A in the exonuclease domain, as well as the mutation A485L in the finger domain. Terminator DNA polymerase was originally developed by NEB to improve the incorporation fluorescent nucleotides for Sanger sequencing. However, we found that under certain conditions, Terminator DNA polymerase functions can also function as an efficient DNA-dependent TNA polymerase.

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Using Terminator DNA polymerase to synthesize TNA on a DNA template, Ichida and Szostak developed a DNA display strategy to generate functional TNA molecules by in vitro selection. This method establishes a genotype-phenotype link by extending a library of self-priming DNA templates with TNA, which allows each TNA sequence to become physically connected to its own DNA message. Using DNA display, we evolved a TNA aptamer with high affinity and high specificity to human thrombin. This demonstration showed that TNA can fold into tertiary structures with ligand binding activity.

In 2013, we developed a two-enzyme replication system for TNA that mimics the natural process of RNA transcription and reverse transcription. This approach was developed to expand the range of evolutionary strategies that could be used to evolve TNA aptamers and catalysts by in vitro selection. This system uses Terminator DNA polymerase to copy DNA into TNA and the Superscript II to copy TNA back into DNA. Using this strategy, TNA replication was limited to a three letter genetic alphabet due to problems associated with the incorporation of tCTP opposite G in the template. When G residues are present in the DNA template, we observe ~30% G to C transversions in the replicated DNA (DNA to TNA to DNA), suggesting that G:G mispairing occurs during TNA synthesis.

The ability to convert genetic information back and forth between DNA and TNA has an enormous impact on biotechnology, molecular medicine, and information storage. This technology could be used to make diagnostic and therapeutic molecules that are extremely resistant to nuclease degradation. It could also be used to store information in a biologically safe medium.

BRIEF SUMMARY

Described herein are modified polymerases, nucleic acids encoding such polymerases, and the use of TNA polymerases for replicating and evolving threose nucleic acids. Specifically, we have engineered a new version of the replicative DNA polymerase 9N (9NRI) that can function in the absence of manganese ions. We used a structure-guided design strategy that involved identifying beneficial mutations from a saturation mutagenesis library that allowed for TNA synthesis in the absence of manganese ions. 9NRI contains the mutations D141A, E143A, A485R, and E664I.

9NRI can synthesize a four nucleotide alphabet (A, T, C, and G) in the absence of manganese ions. Manganese ions decrease polymerase fidelity by altering the active site of the enzyme. If the fidelity of a polymerase is too low, the message would not be copied correctly. The ability to synthesize TNA in the absence of manganese significantly improves TNA transcription fidelity.

9NRI functions with high fidelity as a DNA dependent TNA polymerase. The ability to generate four nucleotide TNA molecules opens the possibility for in vitro selection of complex TNA molecules that are capable of performing complex functions. Additionally, since biologically relevant molecules are usually comprised of four nucleotides, the ability to generate four nucleotide TNA molecules provides potential targeting mechanism for silencing technology. Additionally, there has been much interest in the information storage capabilities of nucleic acids. Since TNA is inherently nuclease resistant, TNA has the potential to become a highly stable and long lasting medium for storing large amounts of information.

9NRI can also synthesize oligonucleotides that have a DNA-TNA mixed sequence backbone with high efficiency

and high fidelity. Chimeric TNA-DNA oligonucleotides provide a convenient strategy for controlled degradation of the oligonucleotide in a biological system. This property could be useful for the development of therapeutic TNA molecules.

Accordingly, in a first aspect disclosed herein is a nucleic acid encoding a TNA polymerase comprising an amino acid sequence at least 95% identical to SEQ ID NO:1, wherein, the residue corresponding to 141 of SEQ ID NO:1 is an alanine, residue 143 is an alanine, residue 485 is an arginine, and residue 664 is an isoleucine; and wherein the encoded DNA polymerase synthesizes a TNA in the presence of a DNA template and threose nucleotides. Other mutations may be possible in the presence of manganese, but RI is the optimal enzyme when looking at just positions 485 and 664. Other enzymes have slightly improved activity as compared to wildtype, but not as good as RI.

In some embodiments the amino acid sequence of the encoded TNA polymerase is at least 98% identical to SEQ ID NO:1. In other embodiments the amino acid sequence of the encoded TNA polymerase comprises the amino acid sequence of SEQ ID NO:1. In some embodiments the amino acid sequence of the encoded TNA polymerase consists of SEQ ID NO:1.

In some embodiments the nucleotide sequence of the nucleic acid comprises SEQ ID NO:2. In other embodiments the nucleotide sequence of the nucleic acid consists of SEQ ID NO:2.

In a related aspect provided herein is a nucleic acid expression vector comprising any of the just-mentioned nucleic acids encoding a TNA polymerase. In a related aspect provided herein is a recombinant cell comprising the foregoing expression vector.

In another aspect provided herein is a purified TNA polymerase comprising an amino acid sequence at least 95% identical to SEQ ID NO:1, wherein, the residue corresponding to 141 of SEQ ID NO:1 is an alanine, residue 143 is an alanine, residue 485 is an arginine, and residue 664 is an isoleucine; and wherein the encoded DNA polymerase synthesizes a TNA in the presence of a DNA template and threose nucleotides.

In some embodiments the amino acid sequence of the purified TNA polymerase comprises an amino acid sequence at least 98% identical to SEQ ID NO:1. In other embodiments the purified TNA polymerase comprises the amino acid sequence of SEQ ID NO:1. In other embodiments the amino acid sequence of the purified TNA polymerase consists of SEQ ID NO:1.

In a related aspect provided herein is a kit comprising any of the above-described TNA polymerases and at least one threose nucleotide (e.g., tA, tT, tG, or tC). In some embodiments the at least one threose nucleotide includes tA, tT, tG, and tC.

In yet another aspect provided herein is a method for synthesizing a TNA, comprising contacting a DNA template with: (i) a TNA polymerase comprising an amino acid sequence at least 95% identical to SEQ ID NO:1, wherein, the residue corresponding to 141 of SEQ ID NO:1 is an alanine, residue 143 is an alanine, residue 485 is an arginine, and residue 664 is an isoleucine; and wherein the encoded DNA polymerase synthesizes a TNA in the presence of a DNA template and threose nucleotides; and (ii) threose nucleotides under conditions that permit TNA polymerization.

In some embodiments the amino acid sequence of the TNA polymerase utilized in the above method is at least 98% identical to SEQ ID NO:1. In other embodiments the

amino acid sequence of the TNA polymerase to be used comprises the amino acid sequence of SEQ ID NO: 1. In other embodiments. In other embodiments the amino acid sequence consists of SEQ ID NO:1.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, and patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIG. 1. DNA and protein sequence of 9N-RI polymerase. 9N-RI contains the following amino acid substitutions with respect to 9N: D141A, E143A, A485R, and E664I.

FIG. 2A. TNA transcription by 9N-RI polymerase of G series templates in the presence and absence of manganese ion. Diagram of the TNA transcription reaction depicting the four different sequences with increasing number of G nucleobases.

FIG. 2B. TNA transcription by 9N-RI polymerase of G series templates in the presence and absence of manganese ion. 9N-RI polymerase produces full-length TNA molecules in both the presence and absence of manganese ions. 9N-RI is the first polymerase capable of producing large quantities of TNA in the absence of manganese ions.

FIG. 3. Fidelity of 9N-RI in the absence of manganese. RI has a fidelity of 99.36% after sequencing 2350 nucleobases.

FIG. 4. 9N-RI polymerase is capable of generating pure TNA products as well as mixed TNA-DNA molecules. The gel on the left depicts the production of pure TNA molecules over time from 5 to 50 hours. The gel on the right depicts chimeric TNA-DNA molecules composed of tA, tG, tT, and dC.

DETAILED DESCRIPTION

Disclosed herein are methods, compositions and systems for replication and in vitro evolution of TNAs based on the unexpected finding that certain TNA synthesis conditions, as described herein, permit the efficient and faithful synthesis of XNAs from DNA templates and their reverse transcription into cDNAs using known polymerases.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein.

In describing the embodiments and claiming the invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "about" means within 5% of a stated range within the relevant parameter.

As used herein, "TNA" or "TNAs" refer to nucleic acids having a backbone composed primarily of α -L-threofura-

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nosyl-(3'→2')(threose)-containing nucleotides, but may include heteropolymers comprising both tNTPs and dNTPs (e.g., dC).

As used herein, "tNTPs" refer to threose nucleotide triphosphates.

As used herein, "tNTP analog" refers to a threose nucleotide triphosphate having a modified base moiety.

As used herein, "TNA polymerase" refers to a polymerase capable of utilizing a DNA template and tNTPs to synthesize a complementary TNA sequence.

With respect to the amino acid sequence homology of polypeptides described herein, one of ordinary skill in the art will appreciate that structural and functional homology of two or polypeptides generally includes determining the percent identity of their amino acid sequences to each other. Sequence identity between two or more amino acid sequences is determined by conventional methods. See, for example, Altschul et al., (1997), *Nucleic Acids Research*, 25(17):3389-3402; and Henikoff and Henikoff (1982), *Proc. Natl. Acad. Sci. USA*, 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff and Henikoff (ibid.). The percent identity is then calculated as: $(\text{[Total number of identical matches]/[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]})(100)$.

Described herein are TNA polymerases, nucleic acids encoding such TNA polymerases, and methods for synthesizing TNAs using DNA as a template. In various embodiments the TNA polymerase comprises an amino acid sequence at least 95% (e.g., 97%, 98%, 99%, or 100%) identical to the amino acid sequence of 9N-RI polymerase shown below as SEQ ID NO:1.

SEQ ID NO: 1; amino acid sequence of Terminator™ DNA polymerase.

```
MILDTDYITENGKPVIRVFKEGFEKIEYDRTFEPYFYALLKDDSAIED
VKKVTAKRHGTVVVKRAEKVQKKFLGRPIEVWKLYFNHPQDVPAIRDRI
RAHPAVVDIYEYDIPFAKRYLIDKGLIPMEGDEELTMLAFATLYHEGE
EPGTGPILMISYADGSEARVITWKKIDLPYVDVSTEEKIMKRLRVVRE
KDPDVLITYNGDNFDAYLKKRCEELGIKFTLGRDGSEPKIQRMGDRFAV
EVKGRIHFDLPVIRRTINLPTYTLEAVYEAVFGKPKVKYAEIEAQAW
SGEGLERVARYSMEDAKVTYELGREFFPMEAQLSRLIGQSLWDVSRSTG
NLVEVFLLRKAYKRNELAPNKPDERELARRGGYAGGYVKEPERGLWDNI
VYLDFRSLYPSIIITHNVSPDTLNRGCKEYDVAPEVGHKFKDFPGFIP
SLGLDLLEERQKIKRKMATVDPLEKKLLDYRQRLIKILANSFYGYGYA
KARWYCKEASVTAWGREYIEMVIRELEEKFGFKVLYADTGLHATIPG
ADAETVKKKAKEFLKYINPKLPGLLELEYEGFYVRGFFVTKKYAVIDEE
GKITTRGLEIVRRDWSEIAKETQARVLEAILKHGDVEEAVRIVKEVTEKL
SKYEVPPEKLVIHEQITRDLRDYKATGPHVAVAKRLAARGVKIRPGTVIS
YIVLKGSGRIGDRAIPADEFDPTKHYDAEYYIENQVLPFAVERILKAFGY
RKEDLRYQKTKQVGLGAWLKVKGKK.
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In some embodiments, the DNA polymerase comprises an A485L point mutation relative to the amino acid sequence of the 9N DNA polymerase and is greater than about 95% identical to the amino acid sequence of Terminator™ DNA

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polymerase (Terminator™ DNA polymerase), e.g., about 96%, 97%, 98%, 99%, or 100% identical to the amino acid sequence of Terminator™ DNA polymerase. In one embodiment, the DNA polymerase to be used comprises the amino acid sequence of SEQ ID NO:1. Typically, TNA synthesis using the Terminator™ polymerase is carried out at about 50° C. to about 60° C. In some embodiments, the TNA synthesis reaction is carried out at about 55° C.

Suitable concentrations of tNTPs range from about 100 μM to about 1000 μM, e.g., about 25, 30, 35, 40, 50, 60, 70, 80, or another concentration of tNTPs from about 100 μM to about 1000 μM.

In some embodiments, the single stranded DNA template to be used in the method comprises a sequence that is restricted to the nucleotides dA, dC, and dT. While not wishing to be bound by theory, it is believed that by limiting single stranded templates to sequences containing these three nucleotides, the fidelity of the sequence transcribed into TNAs is significantly increased as described herein. Also encompassed herein are heteropolymeric TNAs generated by the above-described method, which include tA, tT, tG, and dC.

The fidelity is greatly increased in the presence of Mn if you don't have dG in the templates. These templates transcribe with high efficiency very quickly. Adding the fourth nucleotide to the react greatly reduces the fidelity in the presence of Mn while also reducing its efficiency.

Also described herein is method for reverse transcribing a TNA. In various embodiments, a TNA is reverse transcribed by a method that includes: contacting a TNA template that contains dCTP with a SuperScript II reverse transcriptase in the presence of a primer and dNTPs, and incubating the resulting mix, at a temperature suitable for SuperScript II reverse transcriptase activity, to obtain a cDNA copy of the TNA template. We can also reverse transcribe sequences containing tCTP in the TNA strand. Typically the reverse transcription reaction using the SuperScript II reverse transcriptase is carried out at a temperature of about 37° C. to about 45° C. In some embodiments, the TNA reverse transcription reaction is carried out at 42° C. Also disclosed herein is a method for molecular evolution of threose nucleic acids, which includes the steps of: (i) providing a DNA template library containing diverse DNA template sequences; (ii) hybridizing the template library with one or more complementary primer sequences; (iii) incubating the hybridized template library with a DNA polymerase comprising an amino acid sequence at least 95% (e.g., 97%, 98%, 99%, or 100%) identical to the amino acid sequence of SEQ ID NO:1 in the presence of tTTP, tGTP, tATP, and dCTP, and incubating at a temperature suitable for polymerization by the DNA polymerase to obtain a cTNA library; (iv) subjecting the cTNA library to a selection assay to obtain at least one or more selected cTNAs; and (v) incubating the one or more selected cTNAs with a primer, a SuperScript II reverse transcriptase, and dNTPs at a temperature suitable for SuperScript II reverse transcriptase activity to obtain a selected DNA template library. In some embodiments, the diverse DNA template sequences are restricted to dA, dC, and dT, although the enzyme RI can do all four nucleotides.

TNAs can be selected from a cTNA library in step (iv) based on a number of different criteria and assays depending on a desired functionality or endpoint for the TNAs being generated. Accordingly, in some embodiments the selection assay in step (iv) includes selection of one or more cTNAs from the cTNA library based on affinity for a ligand.

Examples of suitable affinity assays known in the art include, but are not limited to, aptamer affinity chromatography, systematic evolution of ligands by exponential enrichment (SELEX), and kinetic capillary electrophoresis. In other embodiments, selection of one or more cTNAs from the cTNA library is based on a catalytic activity. Methods for assaying and selecting catalytic activities, e.g., ribozyme activities, are known in the art as described in, e.g., Link et al. (2007), *Biol Chem* 388(8):779-786. In some embodiments, one or more cTNAs are selected based on a desired fluorescence emission. See, e.g., Paige et al (2011), *Science*, 333(6042):642-646.

In the various methods described herein, hybridization between a primer and its target sequence is generally carried out under high stringency conditions under which the primer is annealed with its complementary template sequence at a temperature approximately 5° C. below the primer's melting temperature T_m .

Also described herein are TNA transcription systems. In various embodiments a TNA transcription system includes the following components: a single stranded DNA template, a DNA polymerase comprising an amino acid sequence at least 95% identical to the amino acid sequence of Terminator™ DNA polymerase, tTTP, tGTP, tATP; and (i) dCTP; or (ii) a combination of tCTP and dCTP.

Also disclosed herein are TNA reverse transcription systems. Generally a TNA reverse transcription system, as described herein, includes: a TNA template comprising dC, a SuperScript II reverse transcriptase, and dNTPs.

9NRI is a more active TNA polymerase than Terminator polymerase and can be used for all of the same applications as Terminator DNA polymerase. 9NRI is better than Terminator in its ability to transcribe templates of increased complexity with higher efficiency. As a result, researchers can utilize 9NRI to generation more complex molecules with increasingly more sophisticated functions. 9NRI has potential to generate four nucleotide TNA molecules for in vitro selection of complex TNA molecules that are capable of performing complex functions. Additionally, since biologically relevant molecules are usually comprised of four nucleotides, the ability to generate four nucleotide TNA molecules provides potential targeting mechanism for silencing technology. Additionally, there has been much interest in the information storage capabilities of nucleic acids. Since TNA is inherently nuclease resistant, TNA has the potential to become a highly stable and long lasting medium for storing large amounts of information.

EXAMPLES

The invention will be more fully understood upon consideration of the following non-limiting Examples. The invention has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present invention has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, those skilled in the art will realize that the invention is intended to encompass all modifications and alternative arrangements within the spirit and scope of the invention as set forth in the appended claims.

Example 1

Generation of a Mutated DNA Polymerase for TNA Synthesis

In this example, we show how to prepare the mutated DNA polymerase for TNA synthesis. FIG. 1 depicts the nucleotide and amino acid sequence of 9NRI polymerase.

DNA template and its corresponding primer are heated in Thermopol buffer for 5 min at 95 C. The reaction is cooled for 10 min at 4 C to promote the formation of primer-template complexes. After the 10 minute incubation, the enzyme and manganese are combined and added to the primer template complex. The reaction is then brought to 55 C. The reaction is initiated by adding tNTPs to a final concentration of 100 uM each and then incubated for the desired amount of time.

Example 2

Identification of 9NRI

In this example, we describe how we identified and characterized 9NRI and its mutations D141A, E143A, A485R, and E664I. FIG. 2 depicts TNA transcription reactions of 9NRI polymerase in the presence and absence of manganese. Four different templates containing an increase number of sequential G nucleobases were combined with radiolabeled primer, tNTPs, and enzyme in the presence or absence of manganese ions. The reactions were incubated for three hours when in presence of Mn2+ and 25 hrs when in the absence of Mn2+ at 55 C. The TNA transcription reactions were analyzed by denaturing urea polyacrylamide gel electrophoresis. The gels were then imaged on a phosphorimaging screen.

Variants were generated for all 20 amino acids at positions 485 and 664 independently by site directed mutagenesis. Each variant was tested in a TNA transcription reaction and analyzed by polyacrylamide gel electrophoresis. The most active variants at positions 485 and 664 were then generated as double mutants to test for synergistic effects. The double mutants were screened in the exact method as the single mutants.

Example 3

Characterization of 9NRI

In this example, we describe the characterization of the 9NRI enzyme. 9NRI is capable of generating a significant amount of full length TNA in the absence of manganese. Previous enzymes were unable to generate any material. Additionally, 9NRI is capable of transcribing through sequences with higher numbers of sequential G nucleobases.

Example 4

Functions of 9NRI

In this example, we describe how 9NRI can synthesize a four nucleotide alphabet (A, T, C, and G) in the absence of manganese ions and why this is beneficial. 9NRI is capable of generating complex four nucleotide TNA polymers both in the presence and absence of manganese ions. Removing manganese from the transcription reactions greatly improves the fidelity of TNA transcription. The ability to generate four nucleotide TNA sequences enables the selection of biologically-relevant TNA molecules. Four nucleotide sequences have the potential to fold in more complex tertiary structures with more sophisticated functions. Additionally, since most biological genetic materials are composed of four nucleotides, four nucleotide TNA sequences can be generated to target those biomolecules. For example, nucleic acid phar-

maceuticals. TNA's inherent nuclease resistance gives it even more promise for biopharmaceutical development.

Example 5

Fidelity of 9NRI

In this example, we describe how we determined that 9NRI functions with high fidelity as a DNA dependent TNA polymerase. 9NRI was discovered by screening all 20 amino acids at positions 485 and 664. Variants were expressed in XL1-blue competent cells. Clarified cell lysate was used in primer extension reactions in the presence and absence of Manganese ions. Extension efficiency was determined by comparing TNA transcription products by polyacrylamide gel electrophoresis. Single variants with the highest activity were then to look for variant combinations that synergistically improved TNA transcription.

FIG. 3 illustrates the fidelity of TNA transcription in the absence of Mn2+. To determine the fidelity, a DNA template of a fixed sequence was transcribed into TNA using 9NRI polymerase. TNA molecules were then separated from their DNA templates by polyacrylamide gel electrophoresis, cut out of the gel, electroeluted, and then concentrated by ethanol precipitation. Purified TNA molecules were then reverse transcribed into DNA and amplified by PCR. cDNA molecules were then cloned into pJET and analyzed by DNA sequencing. The chart demonstrates that TNA transcription in the absence of Mn2+ proceeds with very high fidelity as compared to in the presence of Mn2+. Removing the Mn2+ results in approximately 12 fold reduction in the error rate of TNA transcription.

Example 6

Efficiency of 9NRI

In this example, we describe how we determined that 9NRI can synthesize oligonucleotides that have a DNA-TNA mixed sequence backbone with high efficiency and high fidelity.

FIG. 4 demonstrates the generation of mixed backbone DNA-TNA chimeric molecules. The substitution of dCTP for tCTP during TNA transcription not only allows for the generation of chimeric molecules, but it also increases the efficiency of TNA transcription. These reactions were performed as in FIG. 2 in the absence of Mn2+. Reactions were sampled every 5 hours and each sampling was analyzed by PAGE and imaged by phosphorimaging.

9NRI was tested for TNA transcription activity with both tCTP and dCTP in the TNA transcription reactions. Products were analyzed by polyacrylamide gel electrophoresis. TNA transcription was more efficient and had a higher fidelity when dCTP was present.

Example 7

Use of 9NRI

In this example, we describe how one of skill would use 9NRI. 9NRI is a highly faithful, highly efficient TNA polymerase. This polymerase enables to synthesis of complex, four-nucleotide TNA polymers. In one embodiment, these polymers can be used to generate libraries of TNA molecules for in vitro selection as well as for generating molecules capable of interacting with naturally occurring biomolecules. Other uses known to one of skill in the art can also apply.

TABLE 1

Sequences of Templates

Name	Sequence
4NT.0G (SEQ ID NO: 3)	5'-ACTATTCAACTTACAATCCTATCAACCTTATAATCCAC TTGGCTACTGCATACGAGTGTC-3'
4NT.1G (SEQ ID NO: 4)	5'-ACTATTCAACTTACAATCGTATCAACCTTATAATCCAC TTGGCTACTGCATACGAGTGTC-3'
4NT.2G (SEQ ID NO: 5)	5'-ACTATTCAACTTACAATGGTATCAACCTTATAATCCAC TTGGCTACTGCATACGAGTGTC-3'
4NT.3G (SEQ ID NO: 6)	5'-ACTATTCAACTTACAATGGGATCAACCTTATAATCCAC TTGGCTACTGCATACGAGTGTC-3'
PBS2 (SEQ ID NO: 7)	5'-GACACTCGTATGCAGTAGCC-3'

The invention has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present invention has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, those skilled in the art will realize that the invention is intended to encompass all modifications and alternative arrangements within the spirit and scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

<210> SEQ ID NO 1

<211> LENGTH: 775

<212> TYPE: PRT

<213> ORGANISM: Thermococcus species 9N-7

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Arg Val Phe Lys Lys Glu Asn Gly Glu Phe Lys Ile Glu Tyr Asp Arg
20 25 30

-continued

Thr	Phe	Glu	Pro	Tyr	Phe	Tyr	Ala	Leu	Leu	Lys	Asp	Asp	Ser	Ala	Ile
		35					40				45				
Glu	Asp	Val	Lys	Lys	Val	Thr	Ala	Lys	Arg	His	Gly	Thr	Val	Val	Lys
	50					55					60				
Val	Lys	Arg	Ala	Glu	Lys	Val	Gln	Lys	Lys	Phe	Leu	Gly	Arg	Pro	Ile
	65				70					75					80
Glu	Val	Trp	Lys	Leu	Tyr	Phe	Asn	His	Pro	Gln	Asp	Val	Pro	Ala	Ile
			85						90					95	
Arg	Asp	Arg	Ile	Arg	Ala	His	Pro	Ala	Val	Val	Asp	Ile	Tyr	Glu	Tyr
			100					105					110		
Asp	Ile	Pro	Phe	Ala	Lys	Arg	Tyr	Leu	Ile	Asp	Lys	Gly	Leu	Ile	Pro
		115					120					125			
Met	Glu	Gly	Asp	Glu	Glu	Leu	Thr	Met	Leu	Ala	Phe	Ala	Ile	Ala	Thr
	130					135					140				
Leu	Tyr	His	Glu	Gly	Glu	Glu	Phe	Gly	Thr	Gly	Pro	Ile	Leu	Met	Ile
	145				150					155					160
Ser	Tyr	Ala	Asp	Gly	Ser	Glu	Ala	Arg	Val	Ile	Thr	Trp	Lys	Lys	Ile
			165						170					175	
Asp	Leu	Pro	Tyr	Val	Asp	Val	Val	Ser	Thr	Glu	Lys	Glu	Met	Ile	Lys
			180					185					190		
Arg	Phe	Leu	Arg	Val	Val	Arg	Glu	Lys	Asp	Pro	Asp	Val	Leu	Ile	Thr
		195					200					205			
Tyr	Asn	Gly	Asp	Asn	Phe	Asp	Phe	Ala	Tyr	Leu	Lys	Lys	Arg	Cys	Glu
	210					215					220				
Glu	Leu	Gly	Ile	Lys	Phe	Thr	Leu	Gly	Arg	Asp	Gly	Ser	Glu	Pro	Lys
	225				230					235					240
Ile	Gln	Arg	Met	Gly	Asp	Arg	Phe	Ala	Val	Glu	Val	Lys	Gly	Arg	Ile
			245						250					255	
His	Phe	Asp	Leu	Tyr	Pro	Val	Ile	Arg	Arg	Thr	Ile	Asn	Leu	Pro	Thr
		260						265					270		
Tyr	Thr	Leu	Glu	Ala	Val	Tyr	Glu	Ala	Val	Phe	Gly	Lys	Pro	Lys	Glu
		275					280					285			
Lys	Val	Tyr	Ala	Glu	Glu	Ile	Ala	Gln	Ala	Trp	Glu	Ser	Gly	Glu	Gly
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Leu	Glu	Arg	Val	Ala	Arg	Tyr	Ser	Met	Glu	Asp	Ala	Lys	Val	Thr	Tyr
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Glu	Leu	Gly	Arg	Glu	Phe	Phe	Pro	Met	Glu	Ala	Gln	Leu	Ser	Arg	Leu
			325						330					335	
Ile	Gly	Gln	Ser	Leu	Trp	Asp	Val	Ser	Arg	Ser	Ser	Thr	Gly	Asn	Leu
			340					345					350		
Val	Glu	Trp	Phe	Leu	Leu	Arg	Lys	Ala	Tyr	Lys	Arg	Asn	Glu	Leu	Ala
		355					360					365			
Pro	Asn	Lys	Pro	Asp	Glu	Arg	Glu	Leu	Ala	Arg	Arg	Arg	Gly	Gly	Tyr
	370					375					380				
Ala	Gly	Gly	Tyr	Val	Lys	Glu	Pro	Glu	Arg	Gly	Leu	Trp	Asp	Asn	Ile
	385				390					395					400
Val	Tyr	Leu	Asp	Phe	Arg	Ser	Leu	Tyr	Pro	Ser	Ile	Ile	Ile	Thr	His
			405						410					415	
Asn	Val	Ser	Pro	Asp	Thr	Leu	Asn	Arg	Glu	Gly	Cys	Lys	Glu	Tyr	Asp
			420					425					430		
Val	Ala	Pro	Glu	Val	Gly	His	Lys	Phe	Cys	Lys	Asp	Phe	Pro	Gly	Phe
		435					440					445			
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	485	490 495
Tyr Gly Tyr Ala Lys Ala Arg Trp Tyr Cys Lys Glu Cys Ala Glu Ser		
	500	505 510
Val Thr Ala Trp Gly Arg Glu Tyr Ile Glu Met Val Ile Arg Glu Leu		
	515	520 525
Glu Glu Lys Phe Gly Phe Lys Val Leu Tyr Ala Asp Thr Asp Gly Leu		
	530	535 540
His Ala Thr Ile Pro Gly Ala Asp Ala Glu Thr Val Lys Lys Lys Ala		
	545	550 555 560
Lys Glu Phe Leu Lys Tyr Ile Asn Pro Lys Leu Pro Gly Leu Leu Glu		
	565	570 575
Leu Glu Tyr Glu Gly Phe Tyr Val Arg Gly Phe Phe Val Thr Lys Lys		
	580	585 590
Lys Tyr Ala Val Ile Asp Glu Glu Gly Lys Ile Thr Thr Arg Gly Leu		
	595	600 605
Glu Ile Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala		
	610	615 620
Arg Val Leu Glu Ala Ile Leu Lys His Gly Asp Val Glu Glu Ala Val		
	625	630 635 640
Arg Ile Val Lys Glu Val Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro		
	645	650 655
Pro Glu Lys Leu Val Ile His Glu Gln Ile Thr Arg Asp Leu Arg Asp		
	660	665 670
Tyr Lys Ala Thr Gly Pro His Val Ala Val Ala Lys Arg Leu Ala Ala		
	675	680 685
Arg Gly Val Lys Ile Arg Pro Gly Thr Val Ile Ser Tyr Ile Val Leu		
	690	695 700
Lys Gly Ser Gly Arg Ile Gly Asp Arg Ala Ile Pro Ala Asp Glu Phe		
	705	710 715 720
Asp Pro Thr Lys His Arg Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln		
	725	730 735
Val Leu Pro Ala Val Glu Arg Ile Leu Lys Ala Phe Gly Tyr Arg Lys		
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<212> TYPE: DNA

<213> ORGANISM: Thermococcus species 9N-7

<400> SEQUENCE: 2

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accgtggtga aagtgaaacg tgcggaaaaa gtgcagaaaa aatttctggg ccgtccgatt    240
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 gacactcgta tgcagtagcc 20

We claim:

1. A purified threose nucleic acid (TNA) polymerase comprising an amino acid sequence at least 95% identical to SEQ ID NO:1, wherein, the residue corresponding to 141 of SEQ ID NO:1 is an alanine, residue 143 is an alanine, residue 485 is an arginine, and residue 664 is an isoleucine; and wherein the encoded DNA polymerase synthesizes a TNA in the presence of a DNA template and threose nucleotides.

2. The TNA polymerase of claim 1, wherein the amino acid sequence is at least 98% identical to SEQ ID NO:1.

3. A kit comprising the purified TNA polymerase of claim 1 and at least one threose nucleotide.

4. The kit of claim 3, wherein the at least one threose nucleotide comprises tA, tT, tG, and tC.

5. A method for synthesizing a TNA, comprising contacting a DNA template with:

- (i) a purified TNA polymerase comprising an amino acid sequence at least 95% identical to SEQ ID NO:1, wherein, the residue corresponding to 141 of SEQ ID NO:1 is an alanine, residue 143 is an alanine, residue 485 is an arginine, and residue 664 is an isoleucine; and wherein the encoded DNA polymerase synthesizes a TNA in the presence of a DNA template and threose nucleotides; and
- (ii) threose nucleotides under conditions that permit TNA polymerization, whereby a TNA is synthesized by the TNA polymerase.

6. The method of claim 5, wherein the amino acid sequence is at least 98% identical to SEQ ID NO:1.

* * * * *